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REVIEW

Alternatives to animal testing: A review

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Abstract The number of animals used in research has increased with the advancement of research and development in medical technology. Every year, millions of experimental animals are used all over the world. The pain, distress and death experienced by the animals during scientific experiments have been a debating issue for a long time. Besides the major concern of ethics, there are few more disadvantages of animal experimentation like requirement of skilled manpower, time consuming protocols and high cost. Various alternatives to animal testing were proposed to overcome the drawbacks associated with animal experiments and avoid the unethical procedures. A strategy of 3 Rs (i.e. reduction, refinement and replacement) is being applied for laboratory use of animals. Different methods and alternative organisms are applied to implement this strategy. These methods provide an alternative means for the drug and chemical testing, up to some levels. A brief account of these alternatives and advantages associated is discussed in this review with examples. An integrated application of these approaches would give an insight into minimum use of animals in scientific experiments.

Contents

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1. Introduction

Use of animals for various purposes like food, transportation, pets, sports, recreation and companionship is as old as the human beings itself. Using animals for the purpose of research is one of the extended uses. Various animals like mice, rats, hamsters, rabbits, fishes (examples – zebra fish, trout), birds (mainly chicken), guinea pigs, amphibians (xenopus frogs), primates, dogs, cats etc. are being used in research for a long time (CULABBR, 1988). Drug testing and toxicological screenings which are useful in the development of new treatments for infectious and non-infectious diseases is the main purpose of such studies. Animals also serve as a tool to understand effects of medical procedures and surgical experiments. Moreover, they are used to obtain products like vaccines, antibiotics etc. which are used in diagnostics as well as treatments (Giacomotto and Segalat, 2010; Hendriksen, 2009, 2007). The number of animals used in research has gone up with the advancement in medical technology. Every year, millions of experimental animals are used all over the world. For example, in UK, 3.71 million animals were used for research in the year 2011 (www.rspca.org.uk). The total number of animals used in the USA in the year 2009 was estimated to be 1,131,076, while that in Germany reached up to 2.13 million in 2001 (Rusche, 2003). This huge population of experimental animals usually comes from the breeding centers located in various universities and national breeding centers. All of these are known as class-A dealers, while the brokers who acquire the animals from miscellaneous sources (like auctions and animal shelters) are identified as class-B dealers. At few instances use of the wild animals such as monkeys and birds is also followed (Baumans, 2005). In clinical testing laboratories, animals are isolated from their groups and used as a tool irrespective of their natural instincts. For the experimental procedures, either a whole animal or its organs and tissues are used. For this purpose animals are euthanized (killed) by established methods. Many times, the animals surviving the clinical testing are euthanized at the end of an experiment to avoid the later pain and distress (Rusche, 2003). In some cases (for example in LD 50 analysis) animals die as a result of the experiment.

The pain, distress and death experienced by the animals during scientific experiments have been a debating issue for a long time. Argument is that being alive, animals have the rights against pain and distress and hence, their use for experimentation is unethical and must be stopped (Rollin, 2003). Various acts and laws have been passed to bring the control over unethical use of animals and minimize the pain to animals during experimentation. For example, in 1824, the organization for animal rights was formed by the Royal Society for the Prevention of Cruelty to Animals. In 1876, an act for prevention of cruelty to animal was formed in the UK (Balls, 1994). It came into existence in India, France and USA in the year 1960, 1963 and 1966, respectively. At present, many rules and acts are followed at the international level, to protect the animals against the cruelty and misuse. The organizations like ICH (International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use), CPCSEA (Committee for Purpose of Control and Supervision on Experiments on Animal), NIH (National Institute of Health), and OECD (Organization for Economic Co-operation and Development) provide the guidelines for animal house keeping, breeding, feeding, transportation, and mainly for their use in scientific experiments (Rollin, 2003). Besides the major concern of ethics, few more disadvantages of animal experimentation are requirement of skilled/trained manpower and time consuming protocols. Moreover, very high cost involved in breeding, housing and lengthy protocols of animal experiments is another drawback (Balls, 1994).

2. Three Rs: reduction, refinement and replacement

Alternatives to animal testing were proposed to overcome some of the drawbacks associated with animal experiments and avoid the unethical procedures. A strategy of 3 Rs is being applied which stands for reduction, refinement and replacement of laboratory use of animals (Ranganatha and Kuppast, 2012). Different methods and alternative organisms are applied to implement this strategy. The concept of replacement of animals was first discussed in 1957 by Charles Hume and William Russell at the Universities Federation for animal welfares (UFAW) (Balls, 1994). Russell and Burch (1959) suggested some ways to make the animal experiments more humanly, which was later called as 3 Rs. This approach motivates the use of minimum number of animals i.e. 'reduction' in the total number of animals used in an experiment. The use of animals must be planned and 'refined' carefully in such a way that pain and distress caused during the experiment should be minimized. Moreover, if possible higher animals should be 'replaced' with alternative methodologies and lower organisms (Ranganatha and Kuppast, 2012; Zurlo et al., 1996). Animal replacement is defined as, 'any scientific method employing non-sentient material which may replace use of conscious living vertebrates in animal experimentation'. Two types of replacements were distinguished as 'relative' and 'absolute' replacement. In relative replacement the animals are used but not exposed to any distress during experiment. No use of

animals at any stage of experiment is identified as the absolute replacement strategy (Balls, 1994).

2.1. Reduction

With the help of statistical support and careful selection of study design one can produce meaningful scientific results of an experiment. For example, *in vitro* cell culture is a good way to screen the compounds at early stages. Use of the human hepatocyte culture gives the information about how a drug would be metabolized and eliminated from the body. Inclusion of such method in study design helps to eliminate unsuitable compounds in preliminary stages only and minimizes the use of animals in further tastings (Kimber et al., 2001). Live animals and embryos are used to study effects of some compounds on embryo development. *In vitro* embryonic stem cell culture test helps to reduce the number of live embryo used and the compounds which are toxic toward developing embryo (Gipson and Sugrue, 1994; De Silva et al., 1996). Also, sharing or providing the discovered data (like characteristics of excipients for the test drug) avoids the necessity of animal studies.

2.2. Refinement

Enriching the cage environment by taking care of animals reduces the stress on animals. Scientists should refine the animal facility so that pain, discomfort and distress during animal life and scientific procedures are reduced. Moreover, under the stress and discomfort there may be imbalance in hormonal levels of animals leading to fluctuations in the results. Hence, experiments need to be repeated which causes an increase in the number of experimental animals. So refinement is necessary not only to improve the life of laboratory animals but also to improve the quality of research (Hendriksen, 2009). For example, it was observed that when mice genetically modified to study Huntington's disease were provided with a complex cage environment with opportunity to nest, hide, gnaw and forage, the disease progressed slowly than the mice in barren cage. Also, such mice were found to mimic the progress of the human disease more closely. Such a refinement provides a very good model to treat the disease and also minimize stress to the animals (De Silva et al., 1996).

2.3. Replacement

Various alternatives to the use of animals have been suggested, such as *in vitro* models, cell cultures, computer models, and new imaging/analyzing techniques (Balls, 2002). The *in vitro* models provide the opportunity to study the cellular response in a closed system, where the experimental conditions are maintained. Such models provide preliminary information for outcome of an experiment *in vivo*. For example, computer models were used to study the working of the heart and to select the potential drug candidates (Gipson and Sugrue, 1994). In many countries, *in vitro* cell cultures have replaced the skin irritancy test and Draize eye irritancy test and use of animals in those. Another example is, extraction of insulin from the pancreas of pigs and cow, but now it is obtained from the bacterial cultures which are lifeline drugs for diabetic patients. This extracted insulin needs to be checked for its purity, efficacy and dose. Use of animals was routine for such checking, but now chromatography techniques are used for checking the purity, efficacy and calculation of dosages of drugs (Foreman et al., 1996). Overall, replacement substantially reduces the use of animals in various processes.

3. Alternative methods

Various methods have been suggested to avoid the animal use in experimentation. These methods provide an alternative means for the drug and chemical testing, up to some levels. Advantages associated with these methods are, time efficiency, requires less man power, and cost effectiveness. These methods are described in detail as follows-

3.1. Computer models

Computers can help to understand the various basic principles of biology. Specialized computer models and software programs help to design new medicines. Computer generated simulations are used to predict the various possible biological and toxic effects of a chemical or potential drug candidate without animal dissection. Only the most promising molecules obtained from primary screening are used for *in vivo* experimentation. For example, to know the receptor binding site of a drug, *in vivo* experimentation is necessary. Software known as Computer Aided Drug Design (CADD) is used to predict the receptor binding site for a potential drug molecule. CADD works to identify probable binding site and hence avoids testing of unwanted chemicals having no biological activity. Also, with the help of such software programs we can tailor make a new drug for the specific binding site and then in final stage animal testing is done to obtain confirmatory results (Vedani, 1991). Hence, the total number of experimental animals is lowered and the objectives of Russel and Burche's 3 Rs are achieved.

Another popular tool is the Structure Activity Relationship (SARs) computer programs. It predicts biological activity of a drug candidate based on the presence of chemical moieties attached to the parent compound. Quantitative Structure Activity Relationship (QSAR) is the mathematical description of the relationship between physicochemical properties of a drug molecule and its biological activity (Knight et al., 2006). The activities like carcinogenicity and mutagenicity of a potential drug candidate are well predicted by the computer database. The recent QSAR software shows more appropriate results while predicting the carcinogenicity of any molecule. The advantages of computer models over conventional animal models are the speed and relatively inexpensive procedures (Matthews and Contrera, 1998). A very good example is a study by Dewhurst et al. (1994) which assessed the effectiveness of computer models versus the traditional laboratory practices. In this comparative study, two groups of undergraduate students performed an experiment with the traditional wet lab approach and computer assisted learning (CAL), respectively. CAL is an interactive computer assisted learning (CAL) program without involvement of real experimental tools. At the end of the study both the groups were assessed for the knowledge gain (through test questionnaires, calculations, and interpretation). It was found that the students performing CAL had a better problem solving attitude. Moreover, the cost of new techniques was much less than the traditional laboratory practices (Dewhurst et al., 1994).

Table 1 Selected examples of organisms as alternatives for laboratory use of animals.

Alternative organism	Remarks
Prokaryotes	
Escherichia coli	Model for molecular and genetic studies
Bacillus subtilis	Model for cellular differentiation
Caulobacter crescentus	
Protists	
Dictyostelium discoideum	Model for molecular and genetic studies
Fungi	
Neurospora crassa	Model for genetic study, circardian rhythm and metabolic regulation studies
Saccharomyces cerevisiae	
Schizosaccharomyces pombe	Model for molecular and genetic studies
Aspergillus nidulans	
Lower vertebrate	
Danio rerio/zebrafish	
Invertebrates	
Amphimedon queenslandica	Studies on evolution, developmental biology and comparative genomics
Aplysia sp./sea slug	Neurobiology
Caenorhabditis elegans	Genetic development studies
Drosophila melanogaster	Genetics and neurology research
Hydra/Cnidaria	To understand the process of regeneration and morphogenesis

3.2. Cells and tissue cultures

Use of *in vitro* cell and tissue cultures which involves growth of cells outside the body in laboratory environment can be an important alternative for animal experiments. The cells and tissues from the liver, kidney, brain, skin etc. are removed from an animal and can be kept outside the body, in suitable growth medium, for few days to several months or even for few years. *In vitro* culture of animal/human cells includes their isolation from each other and growing as a monolayer over the surface of culture plates/flasks. Cellular components like membrane fragments, cellular enzymes can also be used. Various types of cultures like cell culture, callus culture, tissue culture and organ culture are used for various purposes. Benefits associated with techniques are, easy to follow, less time consuming and are less expensive. These methodologies are routinely used for preliminary screening of potential drug molecules/chemicals to check their toxicity and efficacy (Shay and Wright, 2000; Steinhoff et al., 2000). Almost all cosmetics, drugs and chemicals are tested for their toxicity and efficacy, using these tests. For example, eye irritancy test. To check the irritancy of chemicals previously Draize test was used, which requires animals (mainly rabbit). It is very painful and every time a new animal is used. Ke Ping Xu and coworkers suggested an alternative which uses bovine corneal organ culture. The bovine cornea is cultured up to three weeks in laboratory and various analytical methods are used to evaluate the toxicological effect of test chemical irritancy *in vitro* (Xu et al., 2000).

3.3. Alternative organisms

The ethical issues have posed many restrictions over the experimental use of higher model vertebrates like guinea pig, rats, dogs, monkeys etc. Therefore, use of alternative organisms has been proposed. Different model organisms are used to replace experimental animals (Table 1).

3.3.1. Lower vertebrates

Lower vertebrates are an attractive option because of the genetic relatedness to the higher vertebrates including mammals. Moreover, there are less ethical problems involved in the experimental use of lower vertebrates.

3.3.1.1. Example – Danio rerio. *Danio rerio*, commonly called as zebra fish, is a small freshwater fish with an approximate length of 2–4 cm. It has a nearly transparent body during early development, which helps easy visual access to the internal anatomy. The optical clarity allows direct observation of developmental stages, identification of phenotypic traits during mutagenesis, easy screening, assessment of endpoint of toxicity testing and direct observation of gene expression through light microscopy. Small size, short life cycle and high fecundity favor its laboratory use.

The working space, cost of laboratory solutions, test chemicals and the manpower involved are reduced by opting *D. rerio* as an alternative to animals (Hill et al., 2005). Its embryos and larvae can be developed and used for testing in cell culture plates and Petri dishes. Whole genome sequence availability makes Zebra fish an attractive option for molecular and genetic research. From infancy to the adult stage it is used in a variety of applications, mainly for the detection of various toxicological studies of chemicals and pharmaceuticals. It is also having wide applications in the investigation of cancer, heart diseases, neurological malfunctions, behavioral diseases and to observe the mutations and problems in organ development due to exposure to test molecules. Modeling of certain human diseases in zebra fish could be used to ameliorate the disease phenotype and malfunctions in organ development (Peterson et al., 2008).

3.3.2. Invertebrates

Invertebrate organisms are widely used as an alternative for laboratory use of animals. They have been used to study

various diseases like Parkinson's disease, endocrine and memory dysfunction, muscle dystrophy, wound healing, cell aging, programmed cell death, retrovirus biology, diabetes and toxicological testing (Lagadic and Caquet, 1998). Invertebrates have an undeveloped organ system and do not have the adaptive immune system, which poses some limitations for their use in human diseases. However, they hold numerous benefits, such as a brief life cycle, small size and simple anatomy, so that a large number of invertebrates can be studied in a single experiment within a short period with less ethical problems. Their cost of housing is less compared to the animals. For example, thousands of flies could be accommodated in a shelter where only few mice can be kept (Wilson-Sanders, 2011).

3.3.2.1. Example – Drosophila melanogaster. *Drosophila melanogaster*, also known as fruit fly is one of the most widely studied invertebrates in research (Gilbert, 2008). It has a well studied genome which enables study of molecular mechanisms underlying the human diseases. Its complete genome has been sequenced and annotated, which encodes more than 14,000 genes on four chromosomes. Only three genes carry the bulk of genome of *D. melanogaster*. Nearly 75% of the genes involved in human diseases are believed to have a functional homolog in the fly (Reiter et al., 2001; Wilson-Sanders, 2011). *D. melanogaster* requires extremely low cost of maintenance, propagation and screening as compared to the other mammal based models. It also produces the results very rapidly due to a short life cycle. Fruit fly possesses four stages in life cycle – the embryo, the larva, the pupa and the adult. Each stage of fly has its own advantage, hence considered as a multiple model organism to study the various concepts (Pandey and Nichols, 2011). The Embryo is frequently used to study the cell fate determination, neuronal development, axon path finding, organogenesis, fundamental developments and to examine pattern formation. The larva is used to study the physiological and developmental processes and behaviors like foraging. The adult fly is a very complex organism. The functions of various structures like the heart, lungs, gut, kidney and reproductive tract are equivalent as that of mammals (Rothenfluh and Heberlein, 2002).

The response of flies to many drugs which are acting on CNS is similar to that observed in mammals. The brain of the adult fly is quite extraordinary because more than 100,000 neurons form the discreet circuits, which mediate various complex behaviors like circadian rhythms, learning and memory, feeding, sleep, courtship, aggression, grooming and flight navigation (Pandey and Nichols, 2011; Rothenfluh and Heberlein, 2002; Wolf and Rockman, 2008). Number of molecular and genetic tools has been made available to study *Drosophila*. Due to many similarities in development and behavioral activities, fruit fly served as a unique and sensitive model for the study of human genetics and diseases (Beckingham et al., 2005). It is also used to express the protein products found in human diseases and to compare the resulting pathologic conditions. Fruit fly serves as an important tool to investigate neurodegenerative diseases like Alzheimers, Parkinson's, disease and Huntington's disease (Bonini and Fortini, 2003; Iijima and Iijima-Ando, 2008; Iijima et al., 2004). It is used in primary small molecule discovery validation as well as in the target discovery processes by taking advantage of the sophisticated genetics available in it. In 1994 the Nobel Prize for physiology and medicine was awarded to Ed Lewis for his pioneering research defining gene structure in flies, as well as to Eric Weischaus and Christiane Nusslein-Volhard for their studies investigating embryogenesis (Iijima et al., 2004).

3.3.2.2. Example – Caenorhabditis elegans. *Caenorhabditis elegans* is a eukaryotic nematode. This multi cellular organism is approximately 1 mm in length and has a very short generation time. Complete life cycle of this hermaphrodite is about 2–3 weeks. Embryogenesis occurs in 12 h and an adult form is developed in 2.5 days. It is transparent, genetically amenable and has simple cellular complexity. Hence, was selected as a model organism by Nobel laureate Brenner (Barr, 2003; Strange, 2007). Life cycle of *C. elegans* proceeds through various complex developmental stages like embryogenesis, morphogenesis and growth to an adult. This is one of the most commonly used model organisms for research purposes. Information obtained can be applicable to more complex organisms like humans. As a model, *C. elegans* have been used to study various neurological disorders like Huntington's disease, Parkinson's disease, Alzheimer's diseases; various immune disorders as well as cancer, diabetes. It has served development and testing of the therapeutic agents for treatment of these diseases (Artal-Sanz et al., 2006; Faber et al., 1999; Link et al., 2001; Nass et al., 2008; Pujol et al., 2008).

3.3.3. Microorganisms

3.3.3.1. Example – Saccharomyces cerevisiae. Brewing yeast, *Saccharomyces cerevisiae* is the most popular and important model organism due to its rapid growth, ease of replica plating and mutant isolation, dispersed cells, well defined genetic system and highly versatile DNA transformation system. Yeasts can be grown in solid or liquid culture and isolated as colonies derived from a single cell on solid media. The generation time is very short i.e. about 90 min, hence it is very easy to grow a large population and analyze it (Mell and Burgess, 2002). Whole genome of this unicellular fungus has been sequenced in 1996. The nuclear genome contains about 16 chromosomes with more than 13 million base pairs. It also contains an extra nuclear genome in the mitochondria. The budding yeast carries its genetic information in the form of 6000 genes. The number and size of genes are relatively small and the density of genes is very high. Best characterized and studied genome makes *S. cerveisiae* one of the most ideal eukaryotic microorganisms for the biological studies. Presence of similar cellular architecture and rudimentary life cycle like multi cellular eukaryotes is another advantage. The numerous membrane-bound organelles like nucleus, peroxisome, mitochondria and the organelles of secretary pathway also mimic the functions of mammalian cells (Mell and Burgess, 2002). This brewing yeast is used to understand programmed cell death, cell death regulators in humans and is very useful in cancer research (Madeo et al., 2002). *S. cerevisiae* helps to understand the fundamental aspects of cellular biology in neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's diseases by studying the endogenous or heterologous proteins that lay at the root of these diseases (Pereira et al., 2012; Siggers and Lesser, 2008).

4. Conclusion

Animal ethics is an issue as important as the human welfare. More efforts need to be undertaken for effective implementation

of 3 Rs during laboratory use of animals. Various alternatives to animal use have been suggested, which need to be implemented in an effective manner. For this integration of various computer models, bioinformatics tools, *in vitro* cell cultures, enzymatic screens and model organisms are necessary. Use of modern analytical techniques, data acquisition and statistical procedures to analyze the results of alternative protocols can provide dependable outcomes. These integrated approaches would result in minimum involvement of animals in scientific procedures.

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